PROCEEDING

International seminar

STRATEGIES FOR THE CONTROL & PREVENTION OF ZOONOTIC DISEASES

Surabaya, 22-23 June 2010
Tandjung Adiwinata Room
Faculty of Veterinary Medicine Airlangga University

Editor:
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MEMBER OF IKAPI: 001/JTI/95

REPORT OF ORGANIZING COMMITTEE

Dr. Anwar Ma'ruf, M.Kes., DVM
Chairman

Indonesia is the one of countries which has many varieties of plants and animals that spread in all over Indonesia region. The kinds of Indonesian animals are domestic, aquatic, exotic, wild animal. There is posibility of that animals to spread of zoonotic diseases if the relationship beetween man and animal is not in a good control.

During the last decades zoonotics diseases threat has been increasing sharply, therefore control of those contagious diseases is definitely needed to prevent their outbreaks. Various zoonotic diseases spread all over the world such as influenza, rabies, brucellosis, anthrax and toxoplasmosis. Global warming has a big chance to change the characteristic of infectious agent that subsequently causes mutation. Mutation will emerge new strains and varians that many threaten mankind health.

Related to those instances, the aim of International Seminar "New Strategies for the Control and Prevention of Zoonotic Diseases" is organized to anticipate the prevention of zoonotic diseases by production of vaccine candidate and antigen diagnostic preventing infectious diseases.

WELLCOME SPEECH

Prof. Hj. Romziah Sidik, Ph.D., DVM
Dean Faculty of Veterinary Medicine
Airlangga University

Assalamu'alaikum wr. wb.

The honorable Rector of Airlangga University,
and our Distinguish Guest Prof. Zainudin (Executive Director of IMHERE),
Prof. Zamri-Saad, Ph.D., DVM. (University Putra Malaysia),
Prof. Dr. Yoes Prijatna Dachlan, MD., MSc. (Triopical Disease Center Unair),
Mrs. Laksmi Wulandari, MD., Sp.P (K), FCCP (Dr. Soetomo Publich Hospital),
Mr. Regis Vialle (Asia-Pacific Area Manager of Ajuvant Vaccine for Zoonotic),
Mr. Bora Gokylmaz (Bioenginering – PT. Global Haditech, Jakarta),
Mr. Soegiarto, DVM., MSc., Ph.D (Disease Investigation Center, Maros),
Dr. Mustapha Bala Abubakar (University of Maiduguri, Nigeria),
Mr. Anton Soekoco, ST. (PT. Fajar Mas Surabaya),
All Head and Secretary Department of Faculty of Veterinary Medicine Unair, all participants, and students.

Ladies and Gentlement,

On behalf International Seminar Organizing Committee and Faculty of Veterinary Medicine, Airlangga University, I would like to say thank you for your coming to attend this International Seminar on "New Strategies for the Control and Prevention of Zoonotic Diseases". I am very appreciate and pleased to welcome you in Airlangga University, Surabaya.

It is very grateful that Faculty of Veterinary Medicine, Unair hold IMHERE- Project B.2.c component for three years implementations that starting from year 2009 to 2012. The Project Implementation Programs include three activities: (1) Research Development on Rabies, *Avian Influenza* (AI), and *Brucella abortus*; (2) Development of Veterinary Diagnostic Services and Training; (3) Development of Frozen Semen Unit Production. As we know, zoonotic diseases need some extra appointed to do any specific researches to obtain a good strategy for prevention and eradicated it that to protect any transmitted diseases from animals to human. The other way, based on Unair as the Autonomous University, Faculty of Veterinary Medicine must to be have sharing to develop Revenue Generating Activity by Development of Veterinary Diagnostic Services and Training as well as Frozen Semen Unit Production, which are proposing the sustainability and achievement the goals of our faculty vision and mission.

In this event be expressed of increasing information and publication of research result that focus on producing vaccine and diagnostic kit of strategic animal diseases. Again, welcome to the International Seminar and it is great to see so many enthusiastic researchers here to get and bring some innovation and new information about these zoonotic diseases. Please have a nice and enjoy this International Seminar with a great beneficial for us.

In this moment, let me to say a thousand thank you to all speakers, participants, our special guest, and students for your dedicated and patient during follow this International Seminar. I am understand that many of you have traveled from quite a distance and I would like to acknowledge all our distinguish guest.

Billahi Taufiq wal Hidayah,
Wassalammu'alaikum wr. wb.

WELCOME MESSAGE

Prof. Dr. H. Fasich, Apt.
Rector of Airlangga University

Assalamua'alaikum warahmatullahi wabarakatuh

Let us be grateful for God's blessings to us that we all can meet here today. Today we are here to talk and discuss about how to control and prevent zoonotic disease.

As we can gather from the media, infectious disease have not been fully controlled in Indonesia and some other countries. As a consequence, this condition brings some disadvantages to people living in those countries.

For example, failures in controlling such diseases in a country can lead to travel ban and travel warning, which in turn will close various opportunities. This alone is a big disadvantage for the development of the country, including human resource development.

Base on those condition, as well as taking into account Airlangga University role in controlling infectious diseases such as Avian Influenza, this forum is an important step in controlling other infectious diseases.

This is also supported by the fact that many modern diseases, including epidemic ones, started from zoonotic diseases. Even in the modern world, in which people have got sufficient immunity, we should still be aware of zoonotic diseases.

Therefore, in thin very special moment, I would like to invite researchers from all over the world to conduct collaborative research, as specially in tropical diseases, at the hospital of Tropical Infectious Diseases, Located at Airlangga University.

By the end of the year 2010, the hospital is ready to operate and conduct various research on diseases caused by specific microorganism of tropical area. Such research, I believe, will be of beneficial for the development of science and technology.

I certainly hope that collaboration among us will be beneficial in finding a better method for the prevention can control of diseases. This, in turn, will bring a better life for sake of humanity. Thank you very much.

Wassalamu'alaikum warahmatullahi wabarakatuh.

ORGANIZING COMMITTEE

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FACULTY OF VETERINARY MEDICINE AIRLANGGA UNIVERSITY
INTERNATIONAL SEMINAR 22-23 JUNE 2010

'New Strategies for the Control and Prevention of Zoonotic Diseases'

PROGRAM SCHEDULE
FIRST DAY: TUESDAY 22 JUNE 2010

TIME	PROGRAM	
07.30 – 08.30	Registration	
08.30 – 09.00	Opening Ceremony • Traditional Dance • Report: Chairman of the Organizing Committee • Welcome: Dean, Faculty of Veterinary Medicine Airlangga University • Opening: Rector of Airlangga University	
09.00 – 09.30	**Refreshment Break**	
	PLENARY LECTURE-1	
09.30 – 10.00	Keynote Speaker 1: **Dr. Suwarno, M.Si., DVM** (Microbiology Department Faculty of Veterinary Medicine, Airlangga Univ.: Characteristic Rabies Virus in Indonesia).	
10.00 – 10.30	Keynote Speaker 2: **Laksmi Wulandari, dr., Sp.P(K), FCCP.** (RS. Dr. Soetomo, Surabaya: Avian and Swine Flu Outbreak: Surabaya Experience)	
10.30 – 11.00	Keynote Speaker 3: **Prof. Dr. Fedik A. Rantam, DVM.** (Vice President of Indonesia Society for Microbiology: Biology and Characterization of Borna Disease Virus Infection)	
	(Moderator: **Dr. Soelih Estoepangesti, DVM.**)	
	SYMPOSIUM-1	
11.00 – 11.45	Keynote Speaker 4: **Regis Vialle** (Seppic, SA, Perancis: Adjuvant for zoonotic diseases, model specificity?)	
11.45 – 12.45	Keynote Speaker 5: **Bora Gokylmaz** (Bioengineering Switzerland – PT. Global Haditech Jakarta: (Bioequipment for Vaccine Production and Design Engineering Consultant for Pharmaceutical Industry)	
12.45 – 13.15	Discussion (**Moderator: Prof. Dr. Rahayu Ernawati, M.Sc., DVM.**)	
13.15 – 14.15	**Lunch and Prayer Break**	
	FREE PAPER -1/PARALLEL SESSION	
	Small Class Room Lv 1	Tanjung Adiwinata Room
14.15 – 14.25	Presentation AOP 1	Presentation BOP 1
14.25 – 14.35	Presentation AOP 2	Presentation BOP 2
14.35 – 14.45	Presentation AOP 14	Presentation BOP 3
14.45 – 15.00	Discussion: Dr. NMR. Widjaja	Duiscussion: Dr. Garry Cores
15.00 – 15.15	**Refreshment Break**	
15.15 – 15.25	Presentation AOP 4	Presentation BOP 4
15.25 – 15.35	Presentation AOP 5	Presentation BOP 5
15.35 – 15.45	Presentation AOP 6	Presentation BOP 6
15.45 – 16.00	Discussion: Dr. NMR. Widjaja	Discussion: Dr. Garry Cores

SECOND DAY: WEDNESDAY 23 JUNI 2010

TIME	PROGRAM	
	SYMPOSIUM-3	
08.00 – 08.30	Keynote Speaker 6: **Soegiarto, DVM, M.Sc, Ph.D.** (Disease Investigation Center, Maros: Epidemiology of Rabies in Indonesia 2005–2009)	
08.30 – 09.00	Keynote Speaker 7: **Dr. Mustapha Bala Abubakar** (University of Maiduguri, Nigeria: Avian Influenza in Nigeria)	
09.00 – 09.30	Discussion (**Moderator: Dr. Mustofa Helmi Efendi, DVM.**)	
09.30 – 09.45	**Refreshment Break**	
	SYMPOSIUM-4	
09.45 – 10.15	Keynote Speaker 8: **Prof. Zamri Saad, DVM., Ph.D.** (UPM Malaysia: Brucellosis in Malaysia)	
10.15 – 10.45	Keynote Speaker 9: **Prof. Dr.Yoes Prijatna Dachlan, M.Sc., dr.** (Faculty of Medicine, Unair: Parasitic Zoonosis)	
10.45 – 11.15	Discussion (**Moderator: M. Yunus, Ph.D., M.Kes., DVM.**)	
	SYMPOSIUM-5	
11.15 – 11.45	Keynote Speaker 10: **Anton Sukoco, ST.** (PT. Fajar Mas Surabaya, Bio Imaging Navigator: Compound Microscopes in One Box)	
11.45 – 12.00	Discussion (**Moderator: Dr. Dady Soegiarto Nazar, M.Sc., DVM.**)	
12.00 – 13.00	**Lunch and Prayer Break**	
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13.10 – 13.20	Presentation AOP 8	Presentation BOP 8
13.20 – 13.30	Presentation AOP 9	Presentation BOP 9
13.30 – 13.40	Presentation AOP 10	Presentation BOP 10
13.40 – 13.50	Presentation AOP 3	Presentation BOP 14
13.50 – 14.15	Discussion: Dr. Suzanita Utama	Discussion: Boedi S., DVM
14.15 – 14.30	**Refreshment Break**	
14.30 – 14.40	Presentation AOP 11	Presentation BOP 11
14.40 – 14.50	Presentation AOP 12*	Presentation BOP 12
14.50 – 15.00	Presentation AOP 13	Presentation BOP 13
15.00 – 15.10	Presentation AOP 15	Presentation BOP 15
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15.25 – 16.00	Door Prize/Closing Ceremony	

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SPECIFICITY TEST OF KIT LIGAND PROTEIN ISOLATED FROM BOVINE MATURE OOCYTE

Widjiati, Theresia Audita Guretti, Sri Pantja Madyawati, Hana Eliyani
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ABSTRACT

Embryo Transfer was one of reproduction technology used to increase cattle productivity. The succeed of embryo transfer depends on the quality of the embryo. The Quality of oocyte for in vitro fertilization in order to produce in vitro embryo was influenced by the in vitro maturation process. In order to reveal the maximum competence, the growth of the oocyte was also influenced by some factors produced by the follicle which has the function to initiate growth into primary follicle. One of those factors was Kit Ligand. Kit Ligand (KL) was a growth factor bind to C-Kit receptor which was actively involved in initiating the beginning of primordial follicle growth process in the ovary. The lack presence of Kit Ligand could cause malfunction in the oocyte growth. This research consisted of collecting oocyte and in vitro culturing, protein isolation, electro elution to purified Kit-Ligand isolate, producing polyclonal antibody, immunization on experimental animal, and Specification test of Kit Ligand using dot blotting method. The presence of color changing showed specification between the protein as antigen and the polyclonal antibody. Dot blotting method could also confirm the concentration of antibody based on the time difference on collecting sample. The result showed on the second week the immune respond increased. It increased gradually until the fourth week (second bleeding) and then decreased. The production of antibody began to increase again and reach the top on the eighth week (sixth bleeding).

Key words: *kit ligand, embryo transfer, folliculogenesis, dot blotting*

INTRODUCTION

Reproductive disorders in cattle can cause low fertility of the parent resulting in decreased pregnancy rates and the calving rate (Affandhy et al., 2007). In the field of animal husbandry, embryo transfer becomes an alternative development of the reproductive technology to improve livestock productivity. Embryo transfer can increase the birth rate in the recipient so as to increase the number of livestock population (Widjiati, 2007).

Embryo transfer method represents an embryo transfer technique from one parent to another recipient parent. Stages in embryo transfer involve taking an egg cell (ovum), maturation, fertilization, and transfer to the recipient parent. (Mc Donald's, 2001). Application of embryo transfer in cattle has been carried out in Indonesia since a few years ago, but so far the results of conception are still low. One of the main causes of failure of embryo transfer is due to the low quality of embryos *in vitro*. Improving the quality of embryos *in vitro* can be done through the improvement of production management (Putro, 2001).

Embryo transfer technique still has a low success rate. This can be seen from the pregnancy rates that occurred from embryos produced *in vitro* and then transferred to the recipient parent. The low pregnancy rate of embryo transfer technique is partly because the quality of embryos produced *in vitro* is still low. Implementation of the embryo transfer requires embryos *in vitro* with good quality i.e. with a maximum maturity level (Hunter, 2005).

Embryo quality can be improved by taking into account of the spermatozoa and oocyte factors. In laboratory, embryos can be produced regularly and developed to blastocyst stage using three techniques, namely: oocyte in vitro maturation (IVM), followed by sperm capacitation and fertilization of in-vitro matured oocytes (IVF) and then the culture of oocytes that have been fertilized until the blastocyst stage which is called in vitro culture (IVC) (Tanaka et al., 2001; Abdoon, 2001).

The success of oocyte maturation *in vitro* greatly influences the success of *in vitro* fertilization, because only a mature oocyte can be fertilized successfully by a sperm cell (Mahaputra and Mustafa, 2000).

Kit ligand (KL) is a growth factor that can be found in the oocyte granulosa cells bound to the C-Kit receptor located on theca cells. Kit ligand has a role to actively initiate the initial process of primordial follicle development in ovaries. Kit ligand is one of the main factors showed to be involved in the initiation of primordial follicle development (Parrott and Skinner, 1999). *Kit ligand* deficiency causes the follicles do not develop normally (Parrot and Skinner, 1999).

Based on the above background, it is necessary to increase the productivity of livestock. It is also necessary to carry out a research on the character and nature of the Kit ligand protein fraction. The objective of this research is to know the nature of *Kit ligand* proteins by specificity test using dot blotting.

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The study is also intended to determine specificity between Kit ligand proteins with local rabbit polyclonal antibody, immunization result of Kit ligand protein isolates from cattle oocytes matured *in vitro* using dot blotting method. The significance of this research, among others, is to obtain information about the specificity of Kit ligand proteins from bovine oocytes matured *in vitro* so that it can be developed and utilized to improve livestock productivity through improved quality of embryos *in vitro*. In addition, the results of research can also be used by students and other researchers as supporting data for the study of Kit ligand proteins.

METHODS

Collection of Oocytes and Maturation of Oocytes in vitro

Bovine ovaries are obtained from the Slaughter House (RPH) and stored in 0.89% NaCl added with gentamycin sulfate 50 g/ml. Wash them with physiological NaCl solution for several times until the washing solvent is clear and proceed with oocyte aspiration.

Put the collected oocytes into drop medium and wash them, and then incubate them in a CO^2 incubator at 37°C for ± 20 hours to obtain mature oocytes (Widjiati et al, 2008a).

Isolation of Kit Ligand Protein

Oocytes were sampled at 200µl and added with PBS and PMSF Twen 1000 mL. Furthermore, the sample was sonicated for 10 minutes then centrifuged at 10 000 rpm for 15 minutes. Supernatant was added to 500 mL absolute ethanol to precipitate the protein. Then centrifuged again at 6000 rpm for 10 minutes. Add Tris HCl to the pellets with a ratio of 1:1. Store pellets in the freezer. The resulting isolation was a crude protein (crude protein) (Widjiati et al, 2007).

Electro Elution to Purify Kit Ligand Proteins

Put 0.1 M phosphate buffer into the electroeluter. Then the SDS PAGE protein bands based on the marker were cut at the molecular weight 30 to 38 KDa. Protein bands were cut into small pieces and then put them into a cellophane bag. Then put them in 0.2 M phosphate buffer in higher concentration for removing salt. Then they were energized with 250 V, 20 miliAmpere (Aulanni'am, 2005).

Polyclonal Antibody Production

a. Immunization of Experimental Animals and Rabbit Blood Serum Collection

Experimental animals were immunized with the Kit Ligand protein isolate emulsions and Complete Freund's adjuvant (CFA), and booster with injection emulsified with Kit ligand protein isolates and Incomplete Freund's adjuvant (IFA).

Immunizations were done two days after the pre-immune blood sampling with 150 µl Kit Ligand + 150 µl CFA. Five days later the first booster was performed by using 150 mL Kit Ligand + 150 IFA. One week after the first booster, the first blood sampling was done and followed every week over a period of five weeks. The second booster was performed two days after the fifth blood collection with the same dose as the first booster.

Two days after the fifth blood sampling, the second booster was performed by injecting Kit ligand isolates and IFA was added as adjuvant, and then blood was collected five days after the second booster and was repeated every week for five consecutive weeks until the tenth blood sampling (Widjiati et al. 2009).

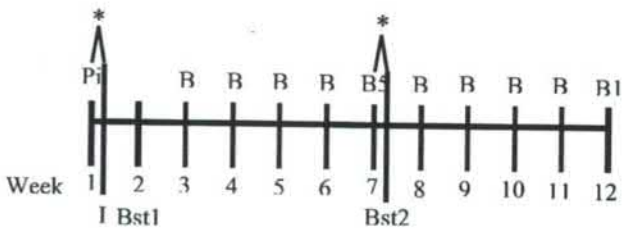

Figure 1. Schema for immunization of experimental animals (Sumedi, 2009)

Pi : Pre-immune blood sampling
I : Immunization using 150 µl *Kit Ligand* + 150 µl CFA
Bst 1,2 : *Booster* 1,2 using 150 µl *Kit Ligand* + 150 µl IFA
$B_1, B_2, \ldots B_7$: Blood sampling in n.. day
* : 2 days

Blood was collected through auricular veins at 5 ml and centrifuged at 3000 rpm for 15 m to obtain serum. Then transfer this serum to Eppendorf tube using pipette and store it at -20°C for dot blotting test.

Specificity Test of Kit Ligand using Dot Blotting Method

Antigen 20 mL was diluted in PBS-sodium azide (NaN3) 1% (1:4) and then dropped on a nitrocellulose membrane that had been soaked in PBS already

assembled to the dot blotter apparatus, gassed* for 30 m. Membrane + antigen were blocked with PBS skim milk 5% for one hour. Discard PBS skim milk and then wash with PBS- Tween 20 0.05% for 3x3 min and incubated in serum/ primary antibodies already diluted in PBS skim milk 5% (1:200) for two hours and shaken gently.

Membrane + Ag-Ab 1 was washed with PBS-Tween 20 0.05% for 3x3 min, and incubated in alkaline phosphatase-labeled secondary serum/antibody (1:2500) that had been diluted in TBS for 1 h while shaken. Membrane + Ag-Ab 1-Ab 2 (AP conjugated) was washed with PBS- Tween 20 0.05% for 3x3 min and incubated in Western Blue substrate for 30 min while shaken and added with distilled water, the membrane was then dried and reading was conducted (Rantam 2003).

RESULTS

The dot blotting demonstrated different colors on nitrocellulose membrane, indicating a presence of the specific binding reaction between antigens and antibodies tested or reacted.

Kit ligand antigen was obtained from ovaries collected from wastes in slaughterhouse. Their oocytes were then collected. Primary antibodies used here were polyclonal antibodies derived from experimental animals immunized with Kit ligand protein antigens so that antibody against the Kit ligand (anti-Kit ligand) was produced. Immunization was done by injecting an antigen and CFA, while the booster was performed by injecting an antigen and the IFA. Antibodies were collected from the blood sampling 10 times and pre-immune weekly. The secondary antibody used was anti-rabbit Ig G which has been labeled with the enzyme alkaline phosphatase.

Besides specificity between protein as antigen and antibody, the dot blotting test also showed that antibody concentration could be confirmed in different harvesting time. This can be seen by comparing several graded blot colors. Scoring below will be useful for interpreting the results.

By making a color scoring from dot blotting results, we can see the increased antibody against Kit Ligand so that peak level of the Kit Ligand antibody can be known. The scoring results above showed a presence of the increased immune response from rabbits (*Oryctolgus cuniculus)* immunized with Kit Ligand.

At the blood sampling in the second week, there was increased immune response gradually until fourth week and then the antibody production decreased. The antibody production began to increase in which production of the Kit Ligand antibody reached peak level at seventh blood sampling.

Regarding the scoring results above, graphics of the average scores from dot blotting can be created as seen below.

Kit Ligand is a protein that can be found in bovine oocytes. A substance can be said to be immunogenic if it has a molecular weight of more than 10 KDa. (Abbas et al., 2000). Kit ligand has a molecular weight of 36 KDa that fulfills the requirement as immunogene. If the Kit ligand is immunized to experimental animals,

Table 1. Dot scoring in nitrocellulose membrane (Sumedi, 2009)

Scoring	Dot scoring in nitrocellulose membrane	Scoring Definition	
		Brightness	Contras
Score 0	Weakly Negative	50%	50%
Score 1	Weakly Positive	51% – 60%	100%
Score 2	Weakly Positive*	61% – 65%	100%
Score 3	Moderately Positive	66% – 75%	100%
Score 4	Moderately Positive*	76% – 82%	100%
Score 5	Strongly Positive	83% – 90%	100%
Score 6	Strongly Positive*	91% – 94%	100%

Note: (*) better quality in the same dot scoring

Tabel 2. Result *dot blotting* protein *Kit Ligand*

	Pengambilan darah ke-								
	1	2	3	4	5	6	7	8	9
Kelinci 1									
Kelinci 2									

can induce the formation of antibodies against the Kit ligand.

First immunization was done with the addition of CFA, which is complete adjuvant that can stimulate antibody production. According to Abbas et al. (2000), the immune response will occur when memory cells have existed before, producing antibodies with high affinity and avidity, and specificity. Therefore, the booster was not performed with CFA, but with IFA which is incomplete adjuvant because immune responses have previously been formed with the first immunization. This is done to prevent the occurrence of hypersensitivity reactions against immunogene.

According to Aulanni'am (2005), the dot blotting test not only shows specificity between protein and antibody, such test also shows that antibody concentration can be confirmed in some different harvesting times.

A change in color on the dot blotting test indicated a presence of antigen binding with antibodies reacted. An antigen is able to recognize its antibody because of the specificity owned by antigen (Auliani'am, 2005). A sample is said positive when it shows color reaction (central point in the sample) with thicker quality compared with the negative control (Lastuti et al., 2006). Negative control in this study was serum from pre-immune blood which has not been injected with antigen. The blotting result in the next blood sampling when compared with the negative control, was positive as demonstrated by a changed color. Thus, we can conclude that there is a specificity between antigen and antibody.

Dot blotting method allows us to know semi-quantitatively a number of the produced antibodies by looking at the antigen-antibody binding (Rantam, 2003). Dot blotting test shows the quality of different colors. This color quality indicates the reaction level between antigen and antibody (Lastuti et al., 2006).

Table 1 shows a color gradation from the dot blotting. Darker color indicates greater antibody concentration. The higher the concentration of antibodies is, the more antigen-antibody binding causing color differences will be.

At the second blood sampling, we can see darker color changes, revealing the elevated antibody production in body of the experimental animal. Antibody production continued to increase gradually up to third week with an average score of 4.5. In the fourth blood sampling, the score reduced to 4 and continued to decline drastically in fifth week with an average score of 2 due to the reduced antibody production.

The second booster was executed two days after the fifth blood sampling. According to Baratawidjaja (2006), the repeated immunization with a certain interval time will enhance immune response in an individual. After the second booster, antibody production began to increase and reached the peak level in the seventh blood sampling with an average score of 6, the highest score. The largest antibody production was reached in this period. After the seventh blood sampling, antibody production dropped again because immune response began to decline significantly.

The reduced antibody production can be seen from the apparent blot color gradation. This is because the amount of the produced antibody decreased substantively and the formed antigen-antibody binding was lower whereby the resulting colors became more pale.

CONCLUSION

Based on the results as mentioned above, we can conclude that there is obvious specificity between Kit ligand proteins and local rabbit polyclonal antibody, immunization result of Kit ligand protein isolates from matured bovine oocytes in vitro by dot blotting method.

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